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Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts

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SUMMARY

Loss of a vimentin network due to gene disruption created viable mice that did not differ overtly from wild-type littermates. Here, primary fibroblasts derived from vimentin-deficient (–/–) and wild-type (+/+) mouse embryos were cultured, and biological functions were studied in in vitro systems resembling stress situations. Stiffness of –/– fibroblasts was reduced by 40% in comparison to wild-type cells. Vimentin-deficient cells also displayed reduced mechanical stability, motility and directional migration towards different chemo-attractive stimuli. Reorganization of collagen fibrils and contraction of collagen lattices were severely impaired. The spatial organization of focal contact proteins, as well as actin microfilament organization was disturbed. Thus, absence of a vimentin filament network does not impair basic cellular functions needed for growth in culture, but cells are mechanically less stable, and we propose that therefore they are impaired in all functions depending upon mechanical stability.

Key words: Cytoskeleton, Collagen lattice, Tensegrity, Mechanotransduction, In vitro wound healing

INTRODUCTION

Vimentin is the major structural component of intermediate filaments in cells of mesenchymal origin, e.g. fibroblasts and endothelial cells (Franke et al., 1987). Based on observations of vimentin expression in particular cell types and developmental stages, different functions, exceeding that of serving a stabilizing role as intracellular scaffold, have been tentatively ascribed to this abundantly expressed cytoskeletal protein. During mouse development, vimentin expression was associated with motility of neural crest (Cochard and Paulin, 1984), parietal endoderm (Lane et al., 1983; Lehtonen et al., 1983), and mesenchymal cells (Franke et al., 1982). Expression in pre-differentiated cell types during the development of the neural system and muscles suggested that it may serve as an early structural scaffold which becomes replaced later by more specialized networks, such as those constituted by glial fibrillary acidic protein (GFAP; Cochard and Paulin, 1984) or desmin (Fürst et al., 1989). Finally, its intracellular distribution has led to the proposal that the vimentin filament system may function as transmission system for mechanical and biochemical signals (Capetanaki et al., 1989; Klymkowsky et al., 1989; Ingber et al., 1994; Maniotis et al., 1997).

However, an overall functional role for vimentin has remained elusive. Addition of vimentin neutralizing antibodies to cultured 3T3 fibroblasts did not induce any major effect on cell shape or physiology (Klymkowsky, 1981; Lin and Feramisco, 1981). Overexpression of intact vimentin in mice leads to aberrant differentiation of the eye lens (Capetanaki et al., 1989a), however, this effect was also observed in mice overexpressing other intermediate filament systems (Monteiro et al., 1990; Dunia et al., 1990). Moreover, two cell lines, of rat and human origin, have been described which, despite lack of a vimentin filament network, grow and proliferate normally in culture with little or no morphological difference (Venetianer et al., 1983; Hedberg and Chen, 1986).

Elucidation of protein function has in recent years entailed targeted inactivation of the corresponding gene and subsequent phenotype analysis of embryos or mice homozygous for the mutation (Thomas and Capecchi, 1987; Koller and Smithies, 1992). Functional deletion of vimentin in mice has unexpectedly not revealed any obvious differences between wild-type and mutant animals with respect to overall development, breeding, structural or functional properties of distinct tissues or organs, including the lens which represents a tissue that expresses vimentin as the sole intermediate

filament component (Colucci-Guyon et al., 1994). In agreement with this result is another report describing that vimentin does not play a role in growth and mobility, and in resistence to mechanical injury (Holwell et al., 1997). First evidence for an abnormal phenotype in these animals caused by vimentin gene inactivation was recently documented for a subset of astrocytes. The lack of a vimentin-based filament network in these cells precludes the formation of an organized GFAP network, which was shown to be restored upon transfection of the cells with a vimentin cDNA construct (Galou et al., 1996).

Thus, despite intense investigation, no clear functions for vimentin could be defined as yet. One of the conclusions to be drawn is that lack of this abundant protein is compatible with life and does not cause an obvious phenotype in culture and even in the whole organism. Possibly any phenotypic manifestations could become obvious only in special conditions, e.g. in stress or pathological situations. Of the different conceivable and in vitro experimentally conductable inductions of stress, such as heat shock, mechanical deformation, sustained exposure to drugs, toxins, mitogens etc., we chose to study cellular functions relevant to the stress situation following tissue injury and leading to wound healing. This decision was based on the fact that a functional cytoskeleton should be a prerequisite for cell motility and migration as well as for contraction and reorganization of connective tissue, all of which are mandatory events for normal wound healing (reviewed by Gailit and Clark, 1994). Apart from this, no studies have been undertaken analyzing the contribution of the vimentin filament network in the dynamic and mechanical events associated with tissue repair, and the present study provides for the first time evidence in primary cultures of mammalian cells of a functional impairment due to lack of vimentin that may bear consequences for tissue repair.

MATERIALS AND METHODS

Cell culture

Primary fibroblasts were obtained from 12.5- or 13.5-day-old homozygous wild-type (+/+) or mutant (–/–) mouse embryos, which were derived from two different targeting events (*Vim1* or *Vim2*; Colucci-Guyon et al., 1994), by dissociation with 0.25% trypsin, 0.02% EDTA in phosphate buffered saline (PBS) and cultured in DMEM with 10% fetal calf serum, supplemented with 300 μg/ml glutamine, 50 μg/ml Na-ascorbate, 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were used for experiments between passages 2 and 8.

In vitro wound closure assay

Fibroblasts were seeded at high density (1×10^6 cells/35 mm culture dishes). Following attachment, one hour after seeding, a scratch was made within the monolayer with a sterile pipette tip. The same spot, indicated by black arrowheads in Fig. 1A, was photographed under phase contrast microscopy (Nikon Diaphot TMD) sequentially at intervals up to 19 hours post 'wounding'. The migrated distance was assessed using 16 measurements per time point and cell strain in 4 independent experiments.

Cell migration and chemotaxis assays

Fibroblast basic motility and chemotactic migration towards conditioned medium (from wild-type cultures), fibronectin (50 μg/ml; Boehringer, Mannheim, Germany) and platelet-derived growth factor (PDGF) AB (30 ng/ml; R & D Systems, Minneapolis, USA) were assayed in standard Boyden chambers using gelatin-coated polycarbonate filters (Costar, 8 μm pore width) according to the method of Albini and Adelmann-Grill (1985). The upper compartment of the chambers was filled with fibroblasts (2.5×10^5/ml), and the lower with chemoattractant. After 4 hours at 37°C, the filters were removed and stained with hematoxylin/eosin (Baxter Diff-Quick, distributed by Dade, Düdingen, Switzerland). Cells which had moved to the bottom side of each filter were counted on 4 non-overlapping squares. Values are derived from 3 independent experiments in duplicate.

Contraction of collagen lattices

Native porcine skin collagen (>95% type I collagen, Deutsche Gelatine Fabriken Stoess AG, Eberbach, Germany) was used to prepare freely contracting collagen lattices in bacteriologic Petri dishes as previously described (Langholz et al., 1995). Fibroblasts were added at cell densities of 0.25×10^5 and 0.5×10^5 per ml. Collagen gel diameters were measured at different time points up to 9 days after onset of the experiments. Rate of gel contraction was expressed as % of remaining surface, calculated from initial surface area set as 100%.

Measurement of cell mechanical stability

Ferromagnetic beads (6 μm diameter, kindly provided by Dr W. Moller, GSF, Gauting, Germany) were coated with a synthetic RGD (arginine-glycine-aspartic acid) -containing peptide (Peptite 2000, Telios, San Diego, CA) at a peptide concentration of 50 μg/mg beads in carbonate buffer and stored at 4°C overnight to facilitate protein adsorption onto the beads (Wang et al., 1993; Wang and Ingber, 1994). Three hours before starting the experiments, wild-type or vimentin-deficient fibroblasts (3×10^4 cells/well) were plated in serum-free medium onto 0.1% gelatin-coated wells (Removawells, Immulon II, Dynatech, Chantilly, VA). RGD-coated beads were added to the wells at 1-2 beads per cell for 15 minutes and unbound beads were washed away with serum-free medium. Each well was submitted to a rotational force in a magnetic twisting cytometer as previously described (Wang et al., 1993). Briefly, the beads were first magnetized by a brief and strong magnetic field (10 microseconds, 1,000 gauss) in the horizontal direction and then twisted by the application of a weaker, homogeneous vertical field (13.3 gauss) that resulted in an apparent stress (torque/bead volume) of 40 dynes/cm^2. Stress was calibrated in a viscosity standard and strain was measured as the beads rotated in response to stress (Wang and Ingber, 1994). The stiffness (defined as the ratio of stress to strain) is a measure of the resistance of the cell to mechanical shape distortion. The twisting forces were also applied after incubation with 1 μg/ml of cytochalasin D for 30 minutes to disrupt the actin network.

In micromanipulation studies, paramagnetic beads (4.5 μm diameter, tosyl-activated, DYNAL Corp.) that were precoated with fibronectin (Ingber, 1990), were added to cells (10 beads per cell) that were cultured on fibronectin-coated glass coverslips. After incubation for 10 minutes at 37°C in a carbon dioxide-buffered incubator, the cells were transferred to an Omega RTD 0.1 stage heating ring coupled to a Nikon Diaphot inverted microscope, and overlayed with a thin layer of mineral oil. Uncoated glass micropipettes were pulled with a Sutter micropipette pulled to produce long tips approximately 1-5 μm wide along a length of 40-100 μm. Beads were mechanically stressed, and ligated cell surface integrins pulled away from the center by juxtaposing the uncoated pipette alongside surface-bound beads and pulling laterally using the micromanipulator.

Immunofluorescence staining of cells

Round glass coverslips placed into the wells of a 24-well plate with or without pre-coating with collagen IV (5 μg/ml) were used as cell culture surfaces. Cells were seeded either for 60 minutes in serum-free DMEM on collagen coats, or for 24 hours in serum containing DMEM on uncoated glass. At the indicated time, adherent cells were fixed with 2% paraformaldehyde in PBS for 15 minutes and permeabilized with 0.2% Triton X-100 for 1 minute. After several PBS rinses, the cells were incubated for 45 minutes with mouse mAb F-VII against human vinculin (a gift from Dr M. Glukhova, Institut Curie, Paris, France), 8d4 against talin (Sigma Immunochemicals, St Quentin-Fallavier, France), or 165 against paxillin (kindly provided by Dr C. Turner, State

University of New York, Syracuse, NY). Indocarbocyanin (Cy3™)-conjugated second antibodies against mouse immunoglobulins (Jackson, distributed through Immunotech, Marseille, France) were applied together with fluorescein isothiocyanate-phalloidin (Sigma Immunochemicals) for another 45 minutes.

The glass coverslips were mounted onto glass slides in 9:1 glycerol/PBS (v/v), and the cells were observed by epifluorescence with a Universal Zeiss microscope (Oberkochen, Germany). Photomicrographs were taken on Kodak T-Max 400 film.

Genotype analysis

The presence of targeted vimentin alleles was monitored using the polymerase chain reaction (PCR) method. A mixture of three oligonucleotide primers was used: primer 1 (5′-TGTCCTCGTCCTCCTACCGC-3′) and primer 2 (5′-AGCTGCTCGAGCTCAGCCAGC-3′) are located upstream and downstream, respectively, of the *Nar*I site in exon 1 of the vimentin gene, primer 3 (5′-CTGTTCGCCAGGCTCAAGGC-3′) is located in the neo^R coding sequence (for details on the targeting constructs, see Colucci-Guyon et al., 1994). The 1-2 primer pair allows amplification of a 398 bp fragment when the wild-type allele is present, and of a 4.8 kb fragment (usually not detected), when the *Vim1* disrupted allele is present. The 2-3 primer pair allows amplification of a 530 bp fragment when the disrupted allele is present. PCR amplification was performed using Taq polymerase (Eurobio, Les Ulis, France) under conditions recommended by the supplier on a Hybaid thermal reactor (Hybaid, Teddington, UK) and involved 30 cycles consisting of 1 minute at 94°C, 1 minute at 65°C, and 1 minute at 72°C.

RESULTS

Fibroblasts were isolated from wild-type (+/+) or vimentin-deficient (–/–) mouse embryos which were generated by targeted gene disruption using two different targeting constructs, pVlacZneo and pVneo, which respectively give rise to *Vim1* and *Vim2* alleles (Colucci-Guyon et al., 1994), and their homozygous genotype was confirmed by genomic PCR analysis (not shown). The cells were maintained in monolayer culture for several passages. As judged by phase contrast microscopy, under these conditions, both strains of mutant fibroblasts appeared flatter than wild-type fibroblasts, the latter displaying the spindle-shape morphology characteristic of

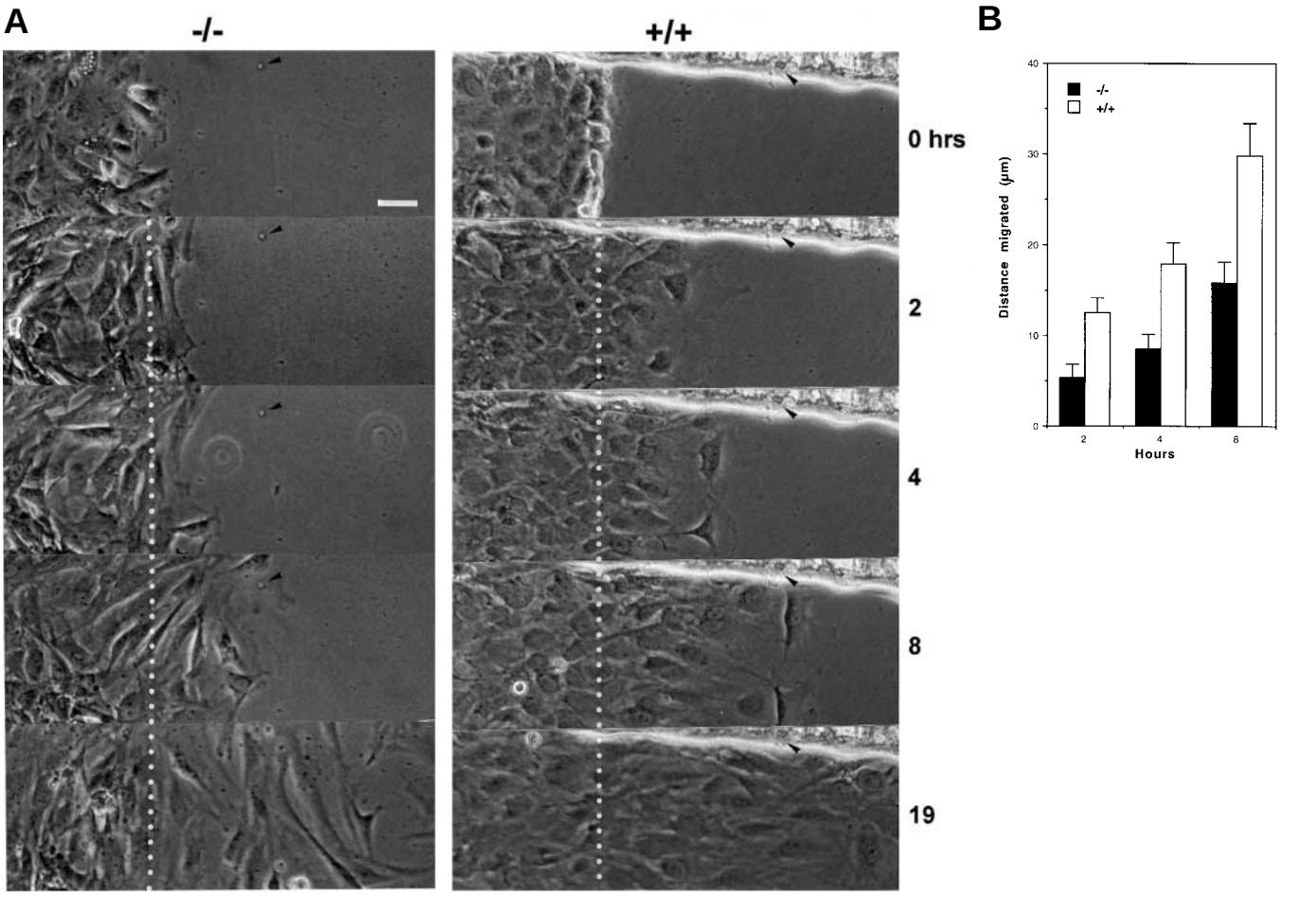


Fig. 1. Delayed migration into a wound of +/+ and –/– mouse embryonic fibroblasts. 1×10^6 wild-type (+/+) and mutant (*Vim1* –/–) fibroblasts were allowed to attach for 90 minutes to 35 mm tissue culture dishes. Using a sterile pipette tip, a 'wound' (clear space) was produced and re-colonization of the cleared space was monitored at different time points at which photographs were taken. (A) Photographs of wounded fibroblast monolayers were taken under phase contrast microscopy at 0, 2, 4, 8, and 19 hours after onset of the experiment. Randomly picked areas to be photographed were marked by a scratch that ran perpendicular to the 'wound' (landmarks indicated by black arrowheads). Bar, 5 μm. (B) The migration of cells (in μm) was measured at 2, 4, and 8 hours after 'wounding'. Values represent mean ± s.d. of 16 measurements per time point, assayed in 4 independent experiments.

embryonal mouse fibroblasts (Fig. 1). Absence of smooth muscle or epithelial cells was confirmed by immunofluorescence staining of cultures for a-smooth muscle actin or keratin expression (not shown). Functionally, *Vim1* –/– and *Vim2* –/– cells consistently gave identical results, as assessed in wound migration and lattice contraction experiments.

Reduced motility and chemotaxis of vimentin-deficient fibroblasts

The behavior of vimentin-deficient and wild-type fibroblasts was compared under conditions which mimic induced movement of cells by creating a wound into the cell monolayer. By mechanical scratching, a space was cleared in the cell monolayer and movement of cells over a period of 19 hours was followed (Fig. 1A). While in wild-type cultures individual cells had detached and started migrating at 2 hours, cells in mutant cultures repopulated the cleared space at a much slower rate (<50%, Fig. 1B). At 19 hours, clearly less mutant cells than wild type had migrated. This result was consistently obtained from both *Vim1* and *Vim2* deficient fibroblasts.

To properly re-colonize a wounded area, cells require both inherent motility and the capacity of directional migration in response to a chemotactic stimulus. In Boyden chamber assays, the random migration of –/– fibroblasts, which reflects inherent cellular motility, was consistently decreased with a mean value of 30% reduction in comparison to +/+ cells (Fig. 2). Differences in directed migration in response to a chemo-attractant gradient were analyzed using conditioned medium derived from wild-type cells, and PDGF and fibronectin which are known to possess high chemo-attractive properties for fibroblasts (Albini and Adelmann-Grill, 1985). Employing these substances, marked differences in chemotactic migration were observed. Although mutant cells showed a distinct propensity to migrate towards all of the chemo-attractants tested, their response was significantly impaired. When compared to controls, chemotaxis of –/– cells to fibronectin, PDGF-AB, or fibroblast conditioned medium, was reduced to, respectively, 40%, 55%, or 53% that of the +/+ counterparts (Fig. 2). In agreement with the results obtained in the in vitro wound healing experiments, deficient cells had a significant decrease in inherent motility (random migration, P=0.0086) as compared to wild type.

Reduced contraction of collagenous lattices by vimentin-deficient fibroblasts

In order to achieve an adequate contraction of the granulation tissue and wound closure following injury, specific cellular mechanical properties are required. We therefore tested the ability of the cells to freely contract three-dimensional collagen lattices, in which collagen fibrils are reorganized by fibroblasts to build a structure resembling connective tissue (Bell et al., 1979).

When seeded within such collagen lattices, vimentin-deficient and wild-type fibroblasts adopted strikingly different morphologies (Fig. 3D) and showed a distinct difference in the magnitude of the contraction forces (Fig. 3A,B,C). Although in –/– collagen gel cultures interconnecting cells could be detected these were, however, much less frequent than in cultures of +/+ cells. Within 1 day, wild-type cells had formed a network with cable-like intercellular connecting cytoplasmic bridges, leading to increased cell numbers per unit area, whereas no such network or increase in cell density could be detected in vimentin-deficient cells cultured within collagen lattices (Fig. 3D).

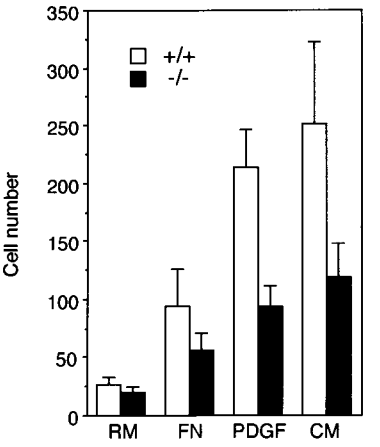


Fig. 2. Inherent motility and chemotactic migration of +/+ and –/– (*Vim1*) mouse fibroblasts. Random migration (RM), and chemotactic migration in response to fibronectin (FN; 50 μg/ml), PDGF-AB (PDGF; 30 ng/ml), and conditioned medium (CM) collected from wild-type monolayer cultures were assayed using 2.5×10^5 cells/ml in standard Boyden chambers. The difference in random migration between wild-type and mutant cells is very significant (P=0.0086, two-sided Student's t-test).

Contraction of collagen lattices was assayed at cell seeding densities of 0.25 and 0.5×10^5 cells/ml. For both cell types, the extent of gel contraction was directly proportional to the initial cell density with most pronounced contraction observed in gels populated by the higher cell density (Fig. 3). However, the contraction forces developed by vimentin-deficient cells (*Vim1* and *Vim2*) were significantly reduced under all conditions tested (Fig. 3A and B). Thus, at 5 days and at the higher cell density, the surface area of +/+ cell-containing gels was reduced to ~5% of the initial surface, while the area of –/– cell-containing gels still amounted to ~50% of the size recorded at the onset of the experiment.

Altered formation of actin network and of focal contacts in vimentin-deficient fibroblasts

Reduced inherent or directed cellular motility of –/– cells, decreased ability to contract collagen gels, as well as altered cell morphology, could be due to modifications of cellular receptors and/or to a defective organization of the contractile apparatus, including the actin fibrils. Visualization of fibrillar actin by staining fibroblasts cultured as monolayers with fluorescein-conjugated phalloidin showed that the actin network was differently organized in +/+ or –/– cells. After 24 hours, distinct actin stress fibers were present in all wild-type fibroblasts, and geodomes resulting from the presence of longitudinal and cortical actin networks were observed in 91% of the +/+ cells (Fig. 4A and C; Table 1). By contrast, in most (~89 %) vimentin-deficient fibroblasts, actin was polymerized into solely longitudinal, thick, and parallel stress fibers (Fig. 4B and D), and geodomes were formed in only 18% of the –/– cells (Table 1).

For closer investigation, the reorganization of the actin network was monitored following the first hour of cell adhesion onto extracellular matrix substrate (Fig. 5). After 60 minutes of

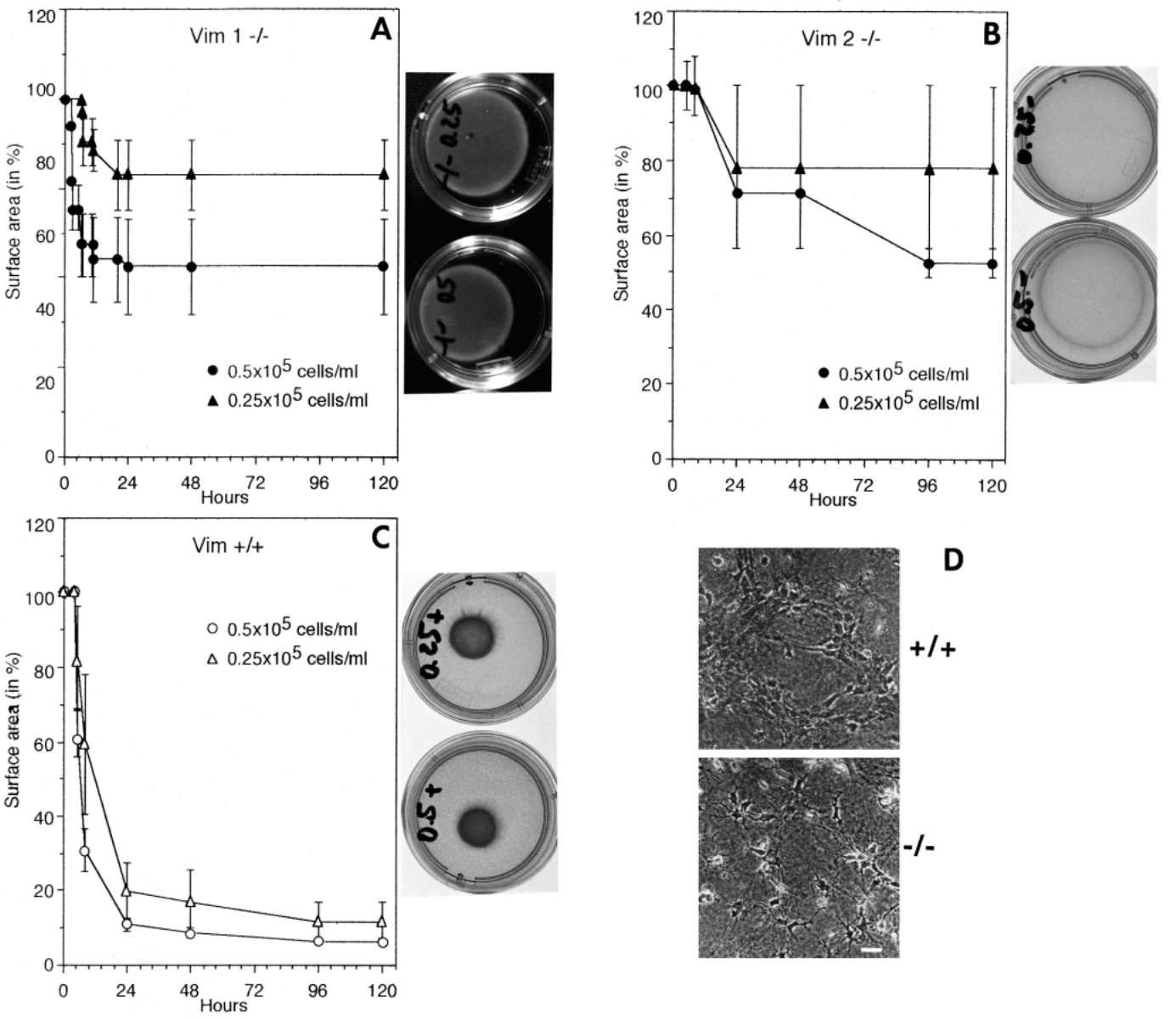


Fig. 3. Contraction of collagen lattices. Graphs show the extent of collagen gel contraction by *Vim1* –/– (A), *Vim2* –/– (B) and wild-type (+/+) (C) fibroblasts seeded at different cell densities as indicated on the figure. Collagen gel diameters were measured at different time points as indicated and the values expressed as % of remaining surface area (surface area at t=0 was set as 100%). Photographs of the contracted collagen lattices at day 9 are shown at the right (top: 0.25×10^5 cells/ml; bottom: 0.5×10^5 cells/ml). (D) Morphology of wild-type and *Vim1* –/– fibroblasts in collagen lattice culture after 24 hours, when compaction of wild-type cultures still allowed phase contrast microscopy. Bar, 16 μm.

adhesion, distinct cortical or longitudinal actin stress fibers were formed in 90% of the +/+ fibroblasts, and the two actin networks were nicely interconnected in most cells (Fig. 5A, micrographs b,f; Table 1). In contrast, fibrillar actin was either not detectable or poorly organized into barely visible microfilaments in 64% of the vimentin-deficient fibroblasts (Fig. 5A, d,h; Table 1). In only 36% of the cells, actin bundles were clearly seen to form either a circular meshwork or longitudinal cables (Fig. 5A, d,h). Very few cells (7%) presented in addition fibrils arranged perpendicular to the cell margins and forming polygonal nets with cortical actin (not shown). In contrast, indirect immunofluorescence staining of several structural components of focal adhesions, including vinculin, talin (Fig. 5A, a,c,e,g), or paxillin (not shown) showed that, although both cell types were capable of clustering these markers into focal contacts, localized to the insertion points of the actin filaments at the periphery or the ventral surface of the cells, there was a difference between +/+ or –/– cells. Observation of the cells at a higher magnification (Fig. 5B) revealed that in wild-type fibroblasts, focal contacts appeared as characteristically well-decorated, individualized patches (Fig. 5B, a,c). In contrast, in mutant cells, decoration of adhesion structures was irregular, and focal contacts were frequently seen as if they failed to remodel into smaller, discrete and separated adhesion complexes (Fig. 5B,

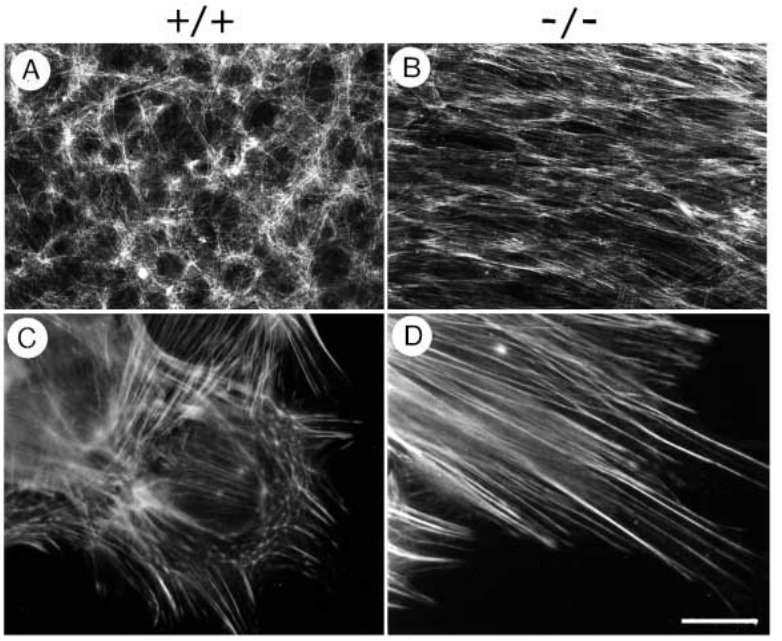


Fig. 4. Immunofluorescence labeling of fibrillar actin in cultivated +/+ and –/– (*Vim1*) cells. Cells were seeded on uncoated glass coverslips in DMEM and 10% FCS for 24 hours, fixed and stained with fluorescein-conjugated phalloidin. The specimen were photographed at low (A,B) and high (C,D) magnification with Kodak T-Max 400 film. –/–: vimentin deficient fibroblasts; +/+: wild-type fibroblasts. Bars: 25 μm (A,B); 5 μm (C,D).

b,d). However, these alterations had no effect on the in vitro capacity of the cells to adhere to extracellular matrix substrates since adhesion to collagens I or IV, fibronectin, or laminin-1 was similar for +/+ or –/– cells (not shown). These data indicate that absence of vimentin has deleterious direct or indirect effects on actin stress fiber organization and anchoring to structures on the inner side of the plasma membrane, and consequently, on the transmission of extracellular forces such as those leading to collagen gel contraction.

Mechanical stability is impaired in vimentin-deficient fibroblasts

For a further detailed analysis of the underlying mechanisms leading to reduced collagen gel contraction, the mechanical stability of the cytoskeleton of vimentin-deficient fibroblasts was analyzed using magnetic twisting cytometry (Wang et al., 1993; Wang and Ingber, 1994). In this technique, controlled mechanical stresses are applied directly to cell surface integrin receptors and interconnected cytoskeletal linkages by magnetically twisting surface-bound magnetic beads that are coated with integrin ligands (synthetic RGD-containing peptide). Using this technique, the mechanical properties of the cytoskeleton can be measured directly in living cells by quantitating the degree of bead rotation. Our results using this technique (Fig. 6E) show that vimentin-deficient cells were about 40% less stiff than wild-type cells, suggesting that vimentin contributes directly to the mechanical stability of the fibroblast cytoskeleton. This observation is in agreement with our earlier finding that cytoskeletal stiffness decreased upon pharmacological inhibition of the intermediate filament system using acrylamide in endothelial cells (Wang et al., 1993). Interestingly, while the disruption of actin microfilaments by cytochalasin D similarly reduced the stiffness of both wild-type and mutant fibroblasts equally by 50-55% of their respective baseline (Fig. 6E), the residual stiffness of the mutant cells was about 30% lower than that of wild-type cells. This result suggests that vimentin is important in stabilizing the residual microtubular cytoskeleton as well.

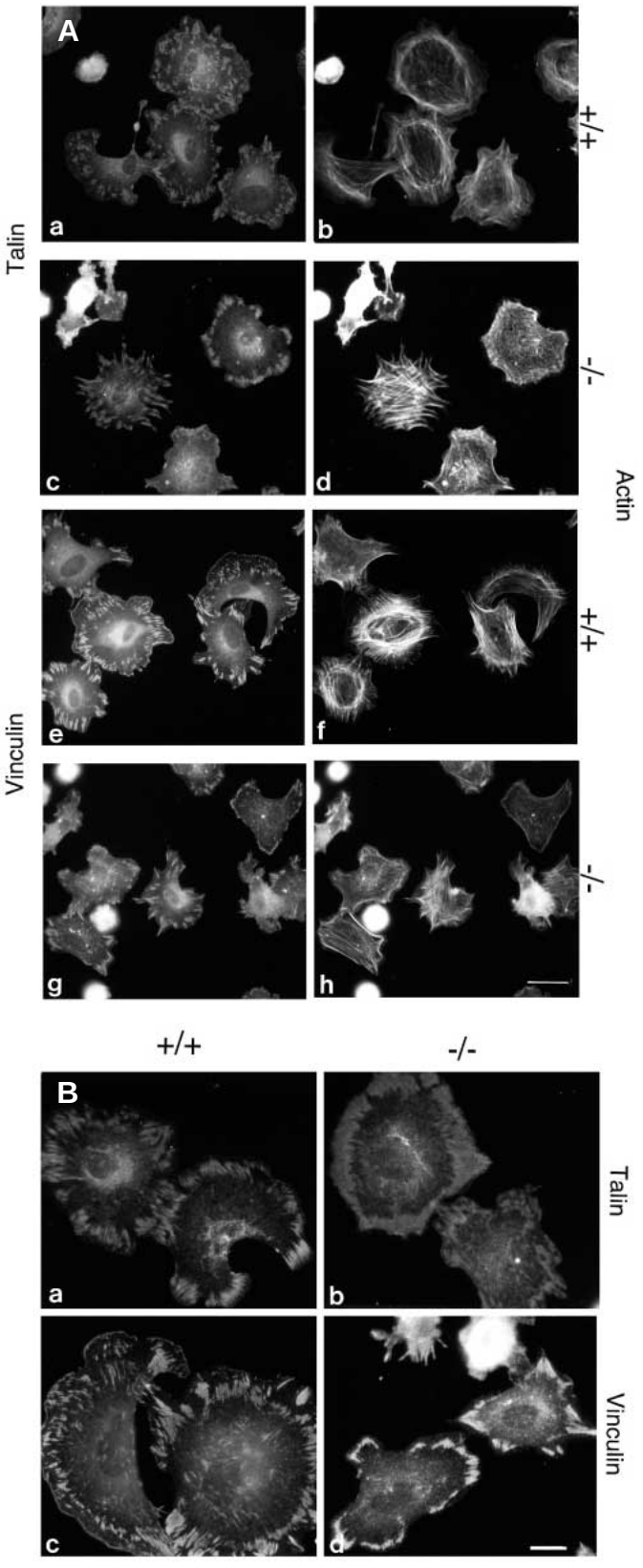
Even more clear demonstration of the importance of vimentin for the mechanical stability of fibroblasts came from studies in which a distending force (rather than a torque) was applied directly to cell surface integrin receptors by using micropipettes and a micromanipulator to pull surface-bound fibronectin-coated microbeads away from the cell surface. When surface-bound beads (4.5 μm diameter) were pulled in wild-type cells, cell surface distension and global cell distortion were observed in the direction of the pull (Fig. 6A,B). In contrast, when surface-bound beads were pulled in a similar manner on vimentin-deficient cells, complete tearing of the cytoplasm including rupture of the plasma membrane resulted (Fig. 6C,D). These results are consistent with the recent demonstration that inhibition of the vimentin system using acrylamide causes similar cytoplasmic tearing and disruption of force transfer between integrins and the nucleus in endothelial cells (Maniotis et al., 1997). Keratin filaments also appear to be necessary to maintain cytoplasmic integrity

Table 1. Architecture of the actin microfilament network after short and long term adhesion

	Cells (%) containing		
	Actin fibers	Actin geodomes	
After 60 minutes			
+/+	90	85	(*n*=88)
–/–	36	7	(*n*=73)
After 24 hours			
+/+	100	91	(*n*=43)
–/–	89	18	(*n*=48)

Cells were seeded either on collagen IV for 60 minutes (conditions are specified in Fig. 6) or on uncoated glass for 24 hours (see Fig. 5), fixed and stained with fluorescein-conjugated phalloidin to visualize fibrillar actin. The number of cells with actin fibers (either cortical, or longitudinal, or both) or with geodomes (cortical *and* longitudinal fibers) are expressed as a percentage of total wild-type (+/+) or vimentin-deficient (–/–) fibroblasts.

in the distending embryonic epithelium during gastrulation (Torpey et al., 1992). Taken together, these results show that vimentin filaments are required for cells to efficiently resist shape distortion (stiffen in response to stress) and to maintain mechanical stability at high levels of mechanical distension (i.e. high strain).



DISCUSSION

We reported previously that complete deficiency of a vimentin filament network, created by targeted gene inactivation in mice, leads to fully viable, normally reproducing animals that display no overt phenotype different from wild-type littermates (Colucci-Guyon et al., 1994). In vivo and in vitro studies showed that GFAP filaments in a subset of astrocytes in these mutant animals failed to assemble into a filament network, suggesting that a preexisting vimentin network is necessary to stabilize GFAP filaments (Galou et al., 1996).

The present report focuses on functional differences observed in cultured primary fibroblasts derived from wild-type and mutant embryos, with particular respect to cytoarchitecture, mechanical properties and interaction with the extracellular matrix. Thus, this report directly contradicts a recent study by Holwell et al. (1997) who detected no differences between vimentin-null and wild-type fibroblasts regarding cell growth, shape, actin and microtubuli networks, motility and resistence to mechanical injury. However, there were two major differences between these authors' experimental scenario and ours: firstly and most importantly, Holwell et al. used vimentin deficient fibroblasts, immortalized by SV40 T antigen expression, a process known to alter a number of cellular functions. Secondly, to test the possible effect of the absence of vimentin, its expression was induced or suppressed via a tetracycline sensitive promoter (Holwell et al., 1997). Although an elegant approach, this system is known to suffer from incomplete regulation, i.e. cells maintained in the continuous presence of tetracycline (necessary to suppress transgene expression in this system) may still exhibit low levels of vimentin expression, which might obscure the biological effects bound to its absence. For the present report, in contrast, primary fibroblasts explanted from either Vim +/+ or Vim –/– embryos were used which had not been subjected to any manipulation except culture.

Cytoarchitecture of vimentin-deficient cells

In our original report on vimentin-deficient mice (Colucci-Guyon et al., 1994) we showed that despite the lack of a vimentin network, microfilaments and microtubular structures appeared normal without an obvious tendency for compensation. These were, however, descriptive data pinpointing a static instant and ignoring the dynamic response of a cell in a tissue. As we could show earlier (Wang et al., 1993; Wang and Ingber, 1994), the mechanical stress forces exerted by a tissue can be measured in vitro by a magnetic twisting device, and the cellular response clearly involves cooperative structural interactions and force

Fig. 5. Double immunofluorescence labeling of talin, vinculin and actin in +/+ and –/– (*Vim1*) cells, 60 minutes after plating. Cells were seeded on collagen IV coated glass coverslips in DMEM in the absence of FCS for 60 minutes. (A) An overview; (B) details of focal contact sites at higher magnification. Adherent cells were fixed and processed for double staining with mouse mAb against talin or vinculin, followed by Cy-3-conjugated goat anti-mouse immunoglobulins, and with fluorescein-conjugated phalloidin to visualize fibrillar actin as indicated in the figure. The specimens were photographed with Kodak T-Max 400 film. –/–: vimentin-deficient fibroblasts; +/+: wild-type fibroblasts. Bars: 8 μm (A); 5 μm (B).

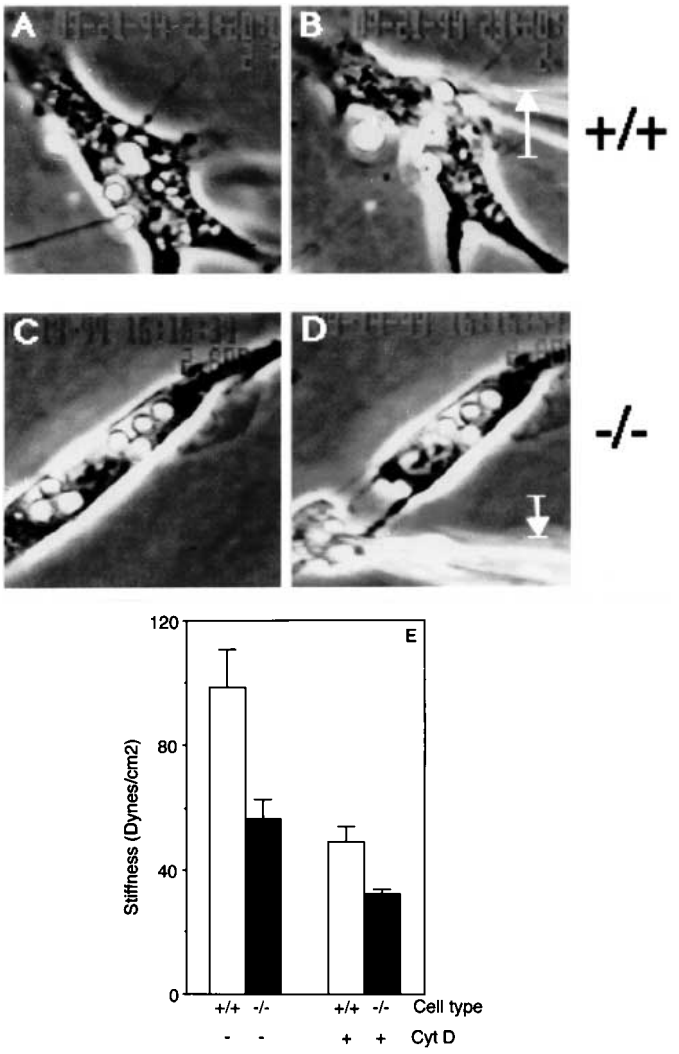


Fig. 6. Mechanical response of wild-type and vimentin-deficient cells. (A-D) Loss of mechanical integrity in the cytoplasm of vimentin-deficient (*Vim1*) fibroblasts. Wild-type (A,B) and vimentin-deficient (C,D) fibroblasts before (A,C) and after (B,D) surface-bound microbeads were rapidly (within 1 second) pulled away from the cell center using an uncoated glass micropipette. Multiple surface-bound beads appear as white, refractile circles (4.5 μm diameter); the white arrow indicates the direction and extent of pipette motion. Note that wild-type cells globally deform in response to local force application, whereas the cytoplasm of vimentin-deficient cells disrupts almost completely even after a small distension (×900). (E) Stiffness in wild-type and vimentin-deficient fibroblasts. Cells were plated and magnetically deformed via integrins as described in Materials and Methods. For selected experiments, fibroblasts were pretreated with cytochalasin D at 1 μg/ml for 15 minutes.

transmission between all three cytoskeletal filament systems. In agreement with these observations, fibroblasts lacking the vimentin intermediate filament system displayed reduced mechanical stability and an inability to support mechanical distension at high strains, such that tearing of the cytoplasm resulted. These results are consistent with the tensegrity model of cytoarchitecture (Wang et al., 1993; Ingber et al., 1994). In this model, intermediate filaments function as ‘tensile stiffeners’ that interlink different structural supports (microfilaments, microtubules, and extracellular matrix tethers) and thereby, stabilize the entire network against shape distortion. In the tensegrity model, loss of these tensile stiffeners will not cause complete disruption of cytoskeletal organization or failure to spread in response to ECM adhesion. Rather, the efficiencies of these responses and the total mechanical strength of the system will be compromised, as we observed here in living vimentin-deficient cells.

The flow of biochemical, positional and mechanical information is thought to proceed via receptors, e.g. integrins, that are connected either directly or indirectly through cytoskeletal linker and adaptor proteins to the actin microfilament system and ultimately to the nucleus (Wang et al., 1993; Ingber et al., 1994; Maniotis et al., 1997). Earlier reports described vimentin to participate in filaments which connect the nucleus with the plasma membrane, and suggested its possible involvement in the formation of focal contacts (Klymkowsky et al., 1989; Green and Goldman, 1986). This possibility has been confirmed in recent micromanipulation studies which show that vimentin filaments can mediate direct mechanical force transfer from cell surface integrins to the nucleus in living endothelial cells (Maniotis et al., 1997). Our observations of an altered spatial arrangement of fibrillar actin and of focal contact associated proteins such as vinculin, talin, or paxillin reflect an aberrant overall cytoskeleton and focal contact architecture in the absence of vimentin filaments. These results, combined with the finding that vimentin-deficient cells physically tear in response to force application to cell surface integrins, provide additional direct support for these earlier findings.

These results are further in agreement with studies by Goldman et al. (1996) who used the identical vimentin-deficient embryonic fibroblasts for microinjection, and found them to be much more fragile and mechanically unstable than wild-type or 3T3 cells. In fact, disruption of vimentin IF network in this report was achieved in vivo in cultured cells by microinjecting mimetic peptides derived from the helix initiation 1A domain of IF protein chains, and changes in cell shape and mechanical stability were very similar to the changes described in our report. The stress fibers in peptide injected cells even appear identical to the –/– cells seen here in Fig. 4 (B and D) with respect to absence of geodome structures.

Overall, more and more evidence has accumulated which indicates that disruption of IF networks results in impaired mechanical stability. This seems to be true for blistering diseases in skin where, due to mutations affecting keratin, keratinocytes have altered cell shapes and intercellular contacts (Fuchs et al., 1994; Steinert et al., 1994; Parry and Steinert, 1995). Further, disruption of desmin, the muscle-specific IF protein, resulted in disruption of muscle architecture and degeneration, in particular affecting skeletal muscle and myocardium (Li et al., 1996; Milner et al., 1996).

These severe physiological consequences are not really surprising when the mechanical and viscoelastic properties of IF in general (Janmey, 1991) and of vimentin in particular (Janmey et al., 1991) are considered. Thus, in contrast to F-actin and microtubules, vimentin filaments are unique in that they become more and more rigid with increasing strain. This observation led to the conclusion that IF are responsible for maintaining cellular integrity.

An earlier report (Bershadsky et al., 1987) had shown, using

double immunofluorescence on quail embryo fibroblasts that vinculin staining in adhesion plaques coincided with the ends of vimentin-positive fibrils. According to this report, formation and maturation of focal contacts are temporally distinct events where microtubules guide intermediate filament attachment to the adhesion plaques, followed then by attachment of microfilament bundles. Thus, temporal organization of focal contacts in vimentin-deficient cells could be altered, resulting in disturbed and possibly malfunctioning structures. Altering the mechanical properties of the cytoskeleton may similarly affect its ability to remodel and strengthen in response to a substrate (Lotz et al., 1989) and, thus, its ability to form focal adhesion structures with normal morphology and distribution.

It is tempting to speculate that the observed disruption of focal contact architecture may play a role in altered transduction of signals in vimentin-deficient fibroblasts. In fact, changes in the expression of some genes, e.g. collagen and fibronectin, MMP-13 were indeed observed (B. Eckes and T. Krieg, unpublished observation), however, the causal relationship is unclear, since tension of a cell by itself is well known to affect gene expression, proliferation, apoptosis and other biological processes (Lambert et al., 1992; Grinnell, 1994).

Migratory and contractile capacities

For most motile cells, including fibroblasts, cell movement is clearly dependent upon the dynamics of an actin microfilament system (reviewed by Lee et al., 1993). The involvement of microtubules is less well studied (reviewed by Schliwa and Höner, 1993). Studies on the participation of intermediate filaments have been scarce, and showed that cells injected with antibodies inducing subsequent collapse of the intermediate filament network, were still able to translocate (Gawlitta et al., 1981). This is in agreement with our observations that also show movement, yet in comparison to the corresponding wild-type cells, vimentin-negative cells displayed reduced motile capacity. According to the tensegrity model of cell migration (described by Ingber et al., 1994), intermediate filaments and microtubules are important because they act to stiffen the internal cytoskeleton and thereby organize polygonal actin networks (e.g. geodomes as shown in Fig. 5 only in wild-type cells) from which filopodia polymerize outward. Thus, although these struts are not inside the lamellipodium, they still may regulate the efficiency of lamellipodia formation and function.

In this context, we were also interested in the question of whether lack of a functional vimentin network might be compensated for by the microtubule or microfilament systems. In view of the participation of all 3 networks in cell movement, migration assays were carried out in the presence of colcemid and cytochalasin D (not shown) which showed comparable levels of reduction in directed migration in both, wild-type and mutant, fibroblasts. These results agree well with the finding that cell stiffness is reduced by cytochalasin D to the same extent in wild-type and mutant cells. This still leaves open the possible involvement of other structures, e.g. proteins that interconnect IF with the microfilament or microtubule system and which were shown to contribute to overall cytoskeletal integrity (Svitkina et al., 1996; Yang et al., 1996), but emphasizes an important function of vimentin in cell motility.

Reduced inherent motility is also thought to be a cause for reduced directional migration. Since the extent of reduction in mutant cells was nearly the same for all chemo-attractants tested, we do not suppose that differences in the corresponding receptors are responsible. Directed migration as assayed here, employing Boyden chambers, could, however, neglect differences in cell deformability which is an important factor for cells tested in crossing the narrow pores of the membrane separating upper and lower compartments in order to maintain a gradient. Our observation of reduced mechanical stability suggests that this may contribute to differences in this assay as well. However, it is difficult to judge whether reduced stability renders cells more flexible, permitting easier movement through the pores, or whether it accounts for more difficult passage.

Possibly the most impressive feature displayed by mutant fibroblasts was their reduced capacity to contract collagen matrices which closely resembled the effect of blocking the collagen integrin receptors (Klein et al., 1991; Langholz et al., 1995; Gotwals et al., 1996). These differences are not due to aberrant expression or functional state of matrix-recognizing integrin receptors, as attachment to laminin-1, collagens I and IV and plastic was identical in wild-type and mutant fibroblasts (not shown). This model which was proposed to represent wound contraction in vitro (Bell et al., 1979) showed clearly a change in mechanical force balance in mutant cells between the forces exerted by the cytoskeleton and opposing those exerted by surrounding collagen fibrils. Viewing the cell as a tensegrity structure (Wang and Ingber, 1994; Ingber et al., 1994) in which microfilaments, microtubules and intermediate filaments form a continuous network that exerts its own tension as well as responds to external tensional forces, our results clearly demonstrate that all three elements contribute, although maybe not equally, to matrix contraction and remodeling. In addition, migratory and contractile capacities are closely linked, as cells contracting a non-anchored matrix need to exert tractional forces by migrating along matrix fibrils (Stopak and Harris, 1982).

In vitro wounding

Although in vitro wound healing, studied in cultured fibroblasts, can only be an approximation to the far more complex biological in vivo events, it represents a valuable experimental system because it is influenced by fewer parameters and therefore yields a clearer picture than in vivo wound healing. Wounding fibroblast monolayers by creating mechanically a cell-cleared space showed that mutant fibroblasts were delayed in filling in the wound defect. This can be attributed to the sum of the malfunctions described above, including reduced mechanical stability, decreased cell motility, and slower directional migration. In addition, it agrees well with studies described by Gurland and Gunderson (1995) who found vimentin interacting with detyrosinated microtubules in the leading edge of migrating fibroblasts at the edge of an in vitro wound, and who proposed that this interaction is generally associated with events such as directed motility.

Collectively, our observations on delayed in vitro wound healing are thought to result from reduced mechanical stability of the cells, caused by the lack of a stabilizing vimentin intermediate filament network. The impaired mechanical stability is thought to lead to decreased cell motility and weaker contractile forces. Whether this is the cause or the consequence of the abnormalities observed in the organization of the fibrillar actin network, in the formation of focal contacts, and possibly in further signal transmission, cannot be decided

at this point. We postulate that vimentin is involved directly or indirectly in all of these processes.

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